FDA-approved Immunosuppressants Targeting Staphylococcal Superantigens: Mechanisms and Insights

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Abstract: Immunostimulating staphylococcal enterotoxin B (SEB) and related superantigenic toxins cause diseases in humans and laboratory animals by hyperactivating cells of the immune system. These protein toxins bind to the major histocompatibility complex (MHC) class II molecules and specific Vβ regions of T-cell receptors (TCR), resulting in stimulation of both monocytes/macrophages and T lymphocytes. The bridging of TCR with MHC class II molecules by superantigens triggers intracellular signaling cascades, resulting in excessive release of proinflammatory mediators and massive polyclonal T-cell proliferation. The early induction of tumor necrosis factor α, interleukin 1 (IL-1), IL-2, interferon γ (IFNγ), and macrophage chemoattractant protein 1 promotes fever, inflammation, and multiple organ injury. The signal transduction pathways for staphylococcal superantigen-induced toxicity downstream from TCR/MHC ligation and interaction of cell surface co-stimulatory molecules include the mitogen-activated protein kinase cascades and cytokine receptor signaling, activating NFκB and the phosphoinositide 3-kinase/ mammalian target of rapamycin pathways. Knowledge of host regulation within these activated pathways and molecules initiated by SEB and other superantigens enables the selection of FDA-approved drugs to interrupt and prevent superantigen-induced shock in animal models. This article focuses on the use of FDA-approved immunosuppressants in targeting the signaling pathways induced by staphylococcal superantigens.

Introduction

Staphylococcal endotoxin B (SEB) and the distantly related toxic shock syndrome toxin 1 (TSST-1) are common etiological agents that cause toxic shock syndrome [1, 2]. The disease is characterized by fever, hypotension, desquamation of skin, and multiple organ system failure [1-3]. These virulence proteins produced by *Staphylococcus aureus* are commonly called "superantigens" as they potently stimulate T-cells, resulting in polyclonal T-cell activation [4-6]. Staphylococcal superantigens hyperactivate cells of the innate immune system and adaptive T-cells concomitantly by binding to the major histocompatibility complex class II (MHC II) molecules on antigen-presenting cells (APC) and specific Vβ regions of T-cell receptors (TCR) [6-14]. However their mode of interaction differs from conventional antigens in that they bind on the outside of the peptide-binding groove of MHC II and exert their biological effects as an intact molecule without being "processed" by APC. In addition, recognition of a superantigen:MHC II complex by the TCR is not MHC-restricted and depends upon the variable region within a TCR β chain (Vβ). Structural properties of many superantigens are well-characterized and most residues involved in their binding to cell surface receptors on immune cells have been identified [14-17]. Various modes of interaction with MHC II and TCRVβ are used by superantigens to promote immunological synapse of interacting cells and cell activation [18, 19]. Activated cells produce cytokines, chemokines, tissue factor, lytic enzymes, and reactive oxygen species, activating both inflammation and coagulation [20-22]. These cytokines include tumor necrosis factor (TNF-α), interferon gamma (IFNγ), and interleukin 1 (IL-1), pro-inflammatory mediators with potent immunoenhancing effects, known to be pathogenic at high levels in vivo [22-28].

Staphylococcal superantigens are stable, single-chain globular proteins of 22- to 30-kD that are highly resistant to proteases and heat denaturation. Despite differences in sequence homology among the staphylococcal enterotoxins (SEs) and TSST-1, they have similarities in their secondary and tertiary structure [6, 29, 30]. Crystallographic studies of staphylococcal superantigens reveal two conserved, tightly packed domains with a β-barrel domain at the N-terminal and a β-grasp motif at the C-terminal. The relatively conserved TCR-binding site is located in the shallow groove between these two domains. Superantigens bind to common, conserved elements outside the peptide-binding groove on MHC II molecules with relatively high affinity [6, 30]. There are at least two distinct binding sites on MHC II molecules for superantigen [11]. A common, low–affinity binding site involving the invariant α-chain of MHC II and a high-affinity, zinc-dependent binding site on the polymorphic β-chain [30-32]. The bridging of superantigen to MHC II and TCR allows cooperative interactions between receptors, hyper-activating the host immune system. Two decades of elegant structural and molecular studies defined binding motifs of bacterial superantigens with MHC II and TCRVβ [6, 30]. Many excellent reviews are available on this topic and would not be discussed further.

The three signals of T-cell activation and signal transduction

Similar to conventional antigen, the binding of superantigen/MHC II to TCR transmits the classical first signal for T-cell activation [33]. Upon superantigen binding, engagement of co-stimulatory molecules CD80 and CD86 on APC with CD28 on T-cells delivers the second signal that optimizes T-cell activation through the formation of stable cell conjugates [34--35]. Other cell adhesion molecules and receptors such as ICAM1 on APC and LFA-1 on T-cells also participate in cell activation by superantigens [36]. Co-stimulatory signaling increases the stability of mRNA of IL-2, IFNγ, TNFα, GMCSF and the expression of anti-apoptotic protein

Bcl-xl to promote T-cell survival. [37-39]. TCR and costimulatory receptors activate protein tyrosine kinases (PTKs), LCK and ZAP-70, resulting in phospholipase C gamma (PLCγ) activation, release of intracellular second messengers and increase in intracellular Ca^{++} [40, 41]. The increase in intracellular calcium concentration activates calcineurin phosphatase which dephosphorylates nuclear factor of activated T-cells (NF-AT) allowing for its translocation into the nucleus where it activates the expression of IL-2 and other T-cell cytokines for T-cell differentiation into TH1 (T helper cell) and other T-cell subsets [33]. Additionally, PTKs also activate protein kinase C (PKC) and Ras GTPase, both of which are also triggered by cell stress and growth factors [33]. The activation of PTK, PLCγ, and PKC initiates three important downstream signaling pathways: (1) Ca^{++}/calcineurin pathway; (2) mitogen–activated protein kinase (MAPK) cascade; and (3) nuclear factor κB (NFκB) pathway, resulting in the activation of transcriptional factors NF-AT, AP-1 (activating protein 1), and NFκB [33, 41-43]. Nuclear NFκB binds to the promoter region of many proinflammatory mediators including IL-1, TNFα, resulting in proinflammatory cytokine expression [43]. The third signal to fully activate T-cells consists of inflammatory cytokines, T-cell growth and differentiation factors, some of which can be induced by signal 1 and 2 [44].

TCR and costimulatory receptor stimulation also activate the lipid kinase, phosphoinositide 3 kinase (PI3K) [45, 46]. Additionally, IL-2 receptor (IL-2R), IFNγR, growth factor receptors, and G-protein-coupled receptor (GPCR) also transduce activation signals via the PI3K pathway upon binding to their respective ligands. PI3K activation generates several inositol phospholipids and activates the protein kinase Akt and mammalian target of rapamycin complex 1 (mTORC1) downstream [45-48]. Activation of mTORC1 leads to phosphorylation and activation of the ribosomal 40S protein p70S6 kinase (p70S6K) and the eukaryotic initiation factor binding

protein 1 (4EBP1) [47, 48]. Phosphorylated p70S6K promotes mRNA translation, protein biosynthesis and cell growth whereas phosphorylation of 4EBP1 enhances protein synthesis by inhibiting its binding to the initiation factor EIF4E. Activation of mTORC1 resulting from all three signals of T-cell activation is essential for G1 to S phase transition as it controls cell proliferation and protein translation [47-49]. Additionally, mTORC1 also functions to integrate diverse signals of nutrient sufficiency and cellular energy via an upstream negative regulator, the AMP-activated protein kinase (AMPK) [49, 50].

Cellular response to superantigens

Human peripheral blood mononuclear cells (PBMC) are often used to study immune cell activation and the subsequenT-cellular changes by superantigens as these cells are responsive to picomolar concentrations of staphylococcal endotoxins (SEs) and TSST-1 [51-54]. The cytokines IL-1, TNFα, IFNγ, IL-2, IL-6, and chemokines, specifically macrophage chemoattractant protein 1 (MCP-1) are induced early by superantigens in human PBMC. There is also a good correlation of the induction of these cytokines with lethal superantigen-induced shock in murine models [24, 26, 28, 55-59]. IL-1 and TNFα also activate other cells including fibroblasts, epithelial, and endothelial cells to perpetuate inflammation by inducing cell adhesion molecules and additional mediators from these cells [60]. Matrix metalloproteases (MMPs) and tissue factor induced by IL-1 and TNFα contribute to the damaging effects on the immune and cardiovascular system, resulting in multi-organ dysfunction and lethal shock. Superantigen-activated T-cells induce the prototypic TH1 cytokine IFNγ which augments immunological responses by increasing MHC class II and adhesion molecule ICAM on APC, epithelial and endothelial cells [26, 36]. IFNγ also upregulates TNFα and IL-1 receptors, thus synergizes with TNFα and IL-1 to promote tissue injury [60]. The T-cell growth factor IL-2 is induced by

superantigen-activated T-cells and promotes T-cell proliferation and differentiation [37, 38]. The receptors and signaling pathways for these mediators are diverse, accounting for the different immunoregulatory activities of cytokines. The intracellular signaling pathways and molecular components of cytokine receptor signaling have been studied extensively as they served as targets of therapeutic interventions.

Cytokines as mediators of inflammation activating NFκB and mTORC1

IL-1interacts with IL-1 receptor 1 (IL-1R1) and an accessory protein to activate NFκB via signaling adaptors myeloid differentiation factor 88 (MyD88), IL-1R-associated protein kinase (IRAK) and TNF receptor associated factor 6 (TRAF6) [60, 61]. This activation pathway is highly conserved and its signaling components are also triggered by the binding of pathogen-associated molecular patterns (PAMPs) to toll-like receptors (TLRs) [62, 63]. PAMPs such as lipoprotein, lipopolysaccharide (LPS), flagellin, dsRNA, and viral RNA bind to specific TLR to activate innate host response. A central component of IL-1R/TLR signaling is the activation of IκB kinases (IKK), resulting in nuclear translocation and activation of NFκB. The MyD88/IRAK/TRAF6 pathway also activates the stress kinase JNK via signaling molecule TRAF6 [64, 65]. SEB upregulates the expression of TLR2 and TLR4, thereby enhancing the host response to other microbial products [66, 67]. This might partially account for the synergistic effects of LPS and SEB in mouse models of SEB-induced shock.

TNFα activates NFκB by binding to TNF receptor 1(TNFR1) or TNFR2. The cytotoxic functions of TNFα are mostly mediated by its binding to TNFR1 via cytoplasmic death domains [68, 69]. The death domain adaptors FADD (Fas-associated death domain), TRADD (TNFR-associated death domain) form a complex with the kinase RIP1 (receptor interacting protein

kinase 1), which then binds TRAF2 to activate the MAPK cascade and NFκB. In addition, the deubiquitylation of RIP1 enables RIP1 to interact with RIP3 to promote necrosis. Activation of death domains by TNFα binding also activates caspase 8 and triggers apoptosis via the extrinsic cell death pathway commonly used by the TNFR superfamily. SEB upregulates the expression of CD95 (Fas), a receptor of the TNFR superfamily, and induces apoptosis via caspase 8 activation [70]. The TNFR superfamily members activate the caspase 8 cascade, JNK, and NFκB, accounting for the pleiotropic effects of TNFα including cell activation, apoptosis, coagulation, inflammation, and host defense [68].

IFNγ (type II IFN) is produced by NK cells, CD8 T-cells and TH1 subset of CD4 T-cells. IFNγ binds to IFNγR and signals via JAK1 (Janus kinase 1), JAK2 and STAT1 (signal transducer and activator of transcription 1) [71-73]. Both type I (IFNα and IFNβ) and type II IFNs signal via PI3K/mTORC1 after binding to two different types of IFN receptors. Although the main function of type I IFN is antiviral, IFNα and IFNβ have overlapping activities with IFNγ as they induce many common interferon-stimulated genes (ISG) [73, 74]. IFNs induce apoptosis and many ISGs have anti-viral, anti-angiogenic and ubiquitylating activities. The immunomodulatory effects of IFNs are mediated by immunity-related GTPase (IRGs) and guanylate binding proteins (GBPs). In addition to antimicrobial defense functions, IFNγ also induces immunoproteasomes and the expression of MHC class II molecules to enhance antigen processing. Similar to IL-1 and TNFα, IFNγ activates PKC leading to MAPK pathway signaling. Both types of IFNs induce and activate death receptors such as CD95, which then activates FADD, subsequently activating caspase 8. Activated caspase 8 can cleave pro-apoptotic molecule Bid to a truncated form allowing for its interaction with two mitochondrial pro-apoptotic molecules, Bak and Bax [75]. The oligomerization of Bak/Bax results in mitochondrial outer-membrane permeabilization, the

release of cytochrome c to the cytosol. Cytochrome c binds cytosolic protein apoptotic protease-activating factor 1 (APAF1) leading to the formation of an apoptosome, a multi-protein complex of APAF1 and caspase 9. Activation of the initiator caspases, caspase 8 for the extrinsic apoptosis pathway by TNFR superfamily or caspase 9 for the intrinsic apoptotic pathway, lead to the induction of caspase 3, caspase 6, caspase 7, and subsequent apoptotic cell death. Damage to mitochondria also releases mitochondrial DNA (mtDNA) which has similar motifs to bacterial DNA and activates intracellular DNA sensors [76-78]. IFNγ increases adhesion molecules on endothelial cells and contributes to vascular cell apoptosis and cardiovascular inflammation [79]. TNFα and IFNγ act synergistically on epithelial cells to increase ion transport and disrupt epithelial barrier function [80, 81]. IFNγ also synergizes with IL-1 and TNFα to promote leukocyte recruitment, inflammation and coagulation [60].

IL-2 is a T-cell growth factor and activates T-cell by binding to high affinity IL-2 receptor. It signals through JAK1 and JAK3, activating PI3K/mTORC1 and Ras to promote cell growth, differentiation and proliferation [82, 83]. Ras activates the MAPK cascade, leading to activation of AP-1 and NFAT. IL-2 from SEB-activated T-cells has potent vascular effects and induces vasodilation, vascular leak, and edema [57, 84, 85]. TNFα synergizes with IL-2 to promote vascular leak as seen in acute lung injury induced by superantigens or pathogens [57, 86].

The chemokines, IL-8, MCP-1, MIP-1α, and MIP-1β, are induced directly by SEB or TSST-1 [26, 87]. Chemokines orchestrate leukocyte migration and activate leukocytes to promote inflammation and tissue injury [60, 88, 89]. Chemokine binds to seven-transmembrane GPCR, induces early calcium flux, activates PLC and signals via the PI3K/mTORC1 pathway [60]. Recruited and activated- neutrophils produce reactive oxygen species (ROS) and MMPs, contributing to organ damage [88]. Either systemic or intranasal exposure to SEB can cause

acute lung injury, characterized by increased expression of adhesion molecules ICAM-1 and VCAM, increased neutrophils and mononuclear cells infiltrate, endothelial cell injury, and increased vascular permeability [57, 84, 85].

Oxidative stress and ROS damage mitochondria

Superantigens induce a massive proliferative response in resting T-cells resembling a mitogenic response. T-cell proliferation requires enhanced protein synthesis and metabolism. Increased glycolysis and fatty acid oxidation support protein biosynthesis but also generate oxidative stress and ROS [90]. Increased protein synthesis, ROS, and activated PKC from cell activation are upstream activators of ER stress [91]. SEB induces the expression of ubiquitin ligases, proteasome peptidases and immunoproteasomes in multiple organs [92]. These ER stress response genes are likely a result of Ca^{++} flux, misfolded proteins and activated PKC. Prolonged ER stress activates the unfolded protein response and apoptosis via the induction of caspases [93, 94]. Increased activity of the mitochondrial electron transport chain following superantigen-activated proliferation also promotes oxidative stress and the generation of ROS, ultimately activating mTORC1.

Increased T-cell proliferation also switches cell metabolism from oxidative phosphorylation to glycolysis and deactivates AMPK, a critical sensor of nutrient and cellular energy, leading to mTORC1 activation [47, 49, 50]. AMPK is a conserved cellular energy sensor activated by decreasing cellular ATP and increasing AMP and ADP. A deleterious consequence of mTORC1 activation is the suppression of autophagy, a homeostatic, catabolic process for the lysosomal degradation of damaged organelles, protein aggregates and intracellular pathogens [95]. Enhanced mitochondrial respiration and ROS damage mitochondria, activate caspase 9 and promote apoptosis [75, 95]. Mitophagy, a special form of autophagy, normally removes damaged

mitochondria resulting from damage and cell stress signals. However, hyperactivation of mTORC1 in superantigen-activated cells disrupts the normal host autophagic removal of damaged mitochondria. Damaged mitochondria release cytochrome c and mtDNA to the cytosol in addition to activating apoptosis via the intrinsic cell death pathway [78]. MtDNA binds endosomal TLR9, activating the transcriptional factors NFκB and IRF7 (interferon-regulatory factor 7). The leakage of mtDNA by damaged mitochondria exacerbates inflammation as mtDNA acts as a potent "damage-associated molecular pattern" (DAMP) to activate cytosolic pattern recognition receptors (PRRs).

DAMPs bind Nod-like receptors (NLRs) activating inflammatory cytokines and pyroptosis

Cytosolic DAMPs such as ROS and mtDNA are upstream activators of inflammasome, an intracellular multi-protein signaling complex that promotes the proteolytic activation of caspase 1 [95-99]. DAMPs bind to intracellular NLRs (nucleotide-binding oligomerization domain (Nod) and leucine-rich repeat-containing receptors) leading to recruitment of the adaptor ASC (apoptosis-associated speck-like protein) which consists of a pyrin domain and a caspase-recruitment domain (CARD). The CARD domain recruits pro-caspase 1 into the inflammasome complex and auto-proteolytic activation of caspase 1 leads to proteolytic processing and activation of proinflammatory cytokines, IL-1β and IL-18. Inflammasome activation also induces pyroptosis, a specialized form of cell death that eliminates cells harboring intracellular pathogens [99]. Other inflammasome activators include lysosomal destabilization, potassium efflux and phagocytosis of bacteria or particulates [98]. ER stress, viral entry and replication can destabilize lysosomes thereby activate inflammasome [100]. Bacteria, bacterial secreted products, viruses, viral DNA and RNA are also potent activators of inflammasome as they bind cytosolic NLRs and induce inflammatory cytokines IL-1, IL-18, and pyroptotic cell death [97-

99]. Apoptosis plays a critical role in down-regulating immune responses but simultaneously has devastating effects when apoptotic cell death is unrestrained. Autophagy is a cellular mechanism that removes bacteria, protein aggregates, and damaged organelles to maintain homeostasis and counteract apoptosis [95, 101, 102]. A recent study indicates that blocking autophagy augments T-cell activation [103]. In superantigen-activated cells autophagy likely contravenes apoptosis as it removes DAMPS and downregulates inflammation.

DAMPs and inflammatory cytokines induce multi-organ injury

IL-1 from inflammasome activation has pleiotropic effects initiating inflammation, NFκB and pyroptosis. TNFα signaling has an established role that initiates cell death, MAPK cascade and NFκB activation. The TNFR superfamily members including TNFR1 and CD95 induce apoptosis by activating caspases and damaged mitochondria also contributes to apoptotic cell death. IFNγ triggers innate host defense responses, antiviral genes, apoptotic programs, immunoproteasomes, and has many immunomodulatory functions. The cell death pathway triggered in vitro and in vivo by superantigens includes genes associated with apoptosis such as FADD, death receptor ligand TRAIL (TNFSF10), caspases, CARD, and PLSCR1 (phospholipid scramblase 1) [92, 104, 105]. These genes are observed in PBMC and major organs from the "double-hit" SEB model and human PBMC stimulated with SEA or SEB. Cellular injury is also apparent from the expression of MMPs, cathepsins, and other cell matrix breakdown products such as versican and fibronectin in superantigen-activated cells [92, 105]. The induction of numerous DNA damage repair enzymes like poly [ADP-ribose] polymerase 9 (PARP9), PARP12, PARP 14 in PBMC and multiple organs of the "double-hit SEB" mouse model indicates DNA damage and repair [92].

Potential drug targets of intervention

There is currently no effective therapeutic treatment for superantigen-induced shock except

for the use of intravenous immunoglobulins [106, 107]. Various humanized monoclonal antibodies are developed to neutralize SEs and TSST-1 by targeting specific epitopes on SEs and TSST-1 [108-111]. However, targeting and neutralizing a superantigen directly is effective only at early stages of exposure before cell activation and initiation of the proinflammatory cytokine cascade.

There are at least three important host-directed targets based on superantigen interaction with host T-cells: (1) TCR and/or MHC class II interactions with toxins; (2) co-stimulatory receptor interactions with toxins; and (3) signaling pathways and molecules induced by activated T-cells and macrophages. Inhibition of all or one of the above three targets/pathways has been reported both in vitro and in vivo, thus representing viable means of blocking the toxic effects of these bacterial superantigens [112]. The targeting of toxin-receptor interaction has been reviewed recently [112]. The disadvantage of this strategy is that to be effective, drugs inhibiting toxin-receptor interaction have to be administered early upon toxin exposure, which is not always possible. Blockade of superantigen activated signal transduction molecules/pathways represents the most amenable mode of intervention as these molecules/pathways occur post-exposure and will likely inhibit other SEs. NFκB and mTORC1 are prime targets in this regard as the three initial signals provided by TCR, costimulatary receptors and cytokines converge on these two hubs of signal transduction. Interruption of these concurrent cascades to tissue injury after superantigens exposure provides an effective strategy in preventing superantigen-induced lethal shock. Many of the superantigen-induced pathways and cell injury are similar to the pathological pathways activated in organ transplantation.

Mouse models of superantigen-induced shock

An obvious component of in vivo testing of therapeutics against superantigen-induced shock is finding a relevant animal model that mimics human disease. Mice are often used as models for obtaining a basic understanding of immunological mechanisms involved in superantigen-mediated shock as reagents such as antibodies againsT-cell surface molecules and mediators are commercially available. However, mice are naturally less susceptible to SEs and TSST-1, compared to humans, because of an inherent lower affinity of these exotoxins for murine MHC class II [113]. Potentiating agents such as D-galactosamine, actinomycin D, or LPS are used to amplify the toxic effects of superantigens [24, 55, 56, 58, 59, 114-116]. These superantigen-induced shock models using potentiating agents have major drawbacks for therapeutic studies as the sensitizing agents themselves often induce the same mediators as SEBs or TSST-1 and activate similar cells and signaling pathways in vivo. Both actinomycin D and D-galactosamine are hepatotoxic and mouse models using these potentiating agents produce unrealistically high levels of TNFα and liver damage [117]. Drugs designed to inhibit TNFα have a higher therapeutic impact in models using these two potentiating agents. In the SEB plus LPS mouse model, the synergistic action of SEB and LPS promotes early TNFα release and prolongs the release of IFNγ, IL-2, IL-6, and MCP-1 [59]. The higher and prolonged levels of these mediators lead to acute mortality with mice succumbing to toxic shock within 48 hours when LPS is used together with SEB [55, 58, 59]. Importantly, the lethal endpoint of these murine models is different from human and non-human primates exposed to SEB [22].

Two newer, simplified murine models have been developed to study SEB-induced shock without potentiating agents. Transgenic mice expressing human MHC class II respond to lower doses of SEB without synergistic agents due to the higher affinity binding of SEB to human MHC class II molecules [11, 22]. Transgenic mice with human HLA-DR3 or -DQ8 lethally

respond to SEs without a potentiating agent and the serum levels of mediators correlate with lethal shock [118-121]. Pathological lesions in lungs of transgenic mice, temperature fluctuations, delayed lethal endpoint later at 96 hour, are similar to those in nonhuman primates exposed to lethal doses of SEB [120]. Low dose continuous administration of SEB to HLA-DQ8 transgenic mice induces a lupus-like syndrome with multiple organ injury [28]. An alternative murine model deploys a "double-hit" strategy with two low doses of SEB using C3H/HeJ mice, an LPS-resistant mouse strain [84]. This "SEB-only" toxic shock model relies on the intranasal administration of SEB and the enhanced action of another dose of SEB strategically spaced 2 hours later in inducing pulmonary inflammation and lethal shock. Importantly, pathological lesions, cytokine response, multiple organ injury and time to lethality in this "SEB-only" model resemble findings in non-human primates and clinical staphylococcal toxic shock syndrome in patients [92]. Gene profiling study in this mouse model with SEB reveals many damage response and IFN-induced genes in multiple organs including (1) innate response; (2) pro- and anti-apoptotic molecules; (3) ER and oxidative stress; (4) intracellular DNA/RNA sensors; (5) immunoproteasome components and E3 ligases; and (6) antiviral ISGs. Upregulation of these damage response genes contributes to irreversible multi-organ damage seen in animal models of toxic shock and human toxic shock syndrome. Many of these genes are also significantly upregulated in SEA- or SEB-induced human PBMC [104, 105].

Repurposing of FDA-approved immunosuppressants

Traditional drug discovery against pathogens and toxins produced by pathogens is a costly and lengthy process with low level of success to FDA approval for human use [122]. The intense investigations to define molecular mechanism of superantigen activation of the immune system present multiple "drugable" targets and pathways. Based on these signaling pathways, an

alternative low cost yet faster approach to target superantigens is drug repurposing. This strategy of drug discovery takes advantage of the known mechanisms of FDA-approved drugs and their safety profile. A dominant signaling hub in superantigen-activated cells is mTORC1 as TCR, CD28, IL-2R, IFNγR and chemokine receptors all signal through the PI3K/mTORC1 pathway. Another key signaling hub is NFκB as TCR and CD28 via PKC also activate NFκB signaling. In addition, proinflammatory cytokines, IL-1 and TNFα, each independently activates NFκB via MyD88/TRAF6/IRAK and FADD/TRADD/RIP, respectively [102]. Activation of NFκB leads to the induction of inflammatory genes, as well as anti-viral, anti-apoptotic and pro-apoptotic molecules seen in the "SEB-only" murine model. Thus the three initial signals provided by TCR, costimulatary receptors and cytokines converge on NFκB and mTORC1. There are some similarities between the pathways leading to the adverse events in transplant rejection and superantigen-induced shock as similar cells and receptors are involved in both types of diseases [42]. Out of the many approaches used against superantigen-induced shock, immunosuppressive agents used to prevent graft loss by suppressing T-cell activation have proven to be the most effective when tested in mouse models [24, 123, 124]. Major classes of FDA-approved immunosuppessants include: (1) Costimulation blockers; (2) NFκB inhibitors; (3) calcineurin inhibitors; and (4) mTORC1 inhibitors. The use of FDA-approved immunosuppressants against staphylococcal superantigens and their mechanism of action are presented in Table 1.

Costimulation blockade

The CD28 costimulatory receptor binds CD80 or CD86 on APC and generates signal 2 for T-cell activation. A transmembrane molecule homologous to CD28, cytotoxic T lymphocyte antigen-4 (CTLA4), is upregulated during T-cell activation and acts as a negative regulator to control T-cell responses [34]. The higher affinity of CTLA4 for CD80 and CD86 enables it to compete

with CD28 for the binding to these costimulatory molecules and block costimulation. A synthetic fusion protein, CTLA4-Ig, inhibits CD28 signaling and prevents lethal TSS by inhibiting costimulation in a D-galactosamine-sensitized mouse model [123]. Blockade of the CD28 by CTLA4-Ig effectively inhibits TSST-1- induced T-cell proliferation, TNFα and IFNγ production in vitro and in vivo [123]. A recent study shows that CTLA4-Ig promotes regulatory T-cell (Treg) development and function in a TGFβ-dependent manner [125]. Thus blockade of the CD28-CD80/86 costimulatory pathway not only blocks costimulation and immunological synapse formation [126] but might also enhance immunosuppression by increasing Treg activity. Two versions of CTLA4-Ig, abatacept and belatacept, are FDA-approved biologics for rheumatoid arthritis and prevention of renal transplant rejection, respectively, but have not been tested against superantigen in animal models [127, 128].

NFκB inhibitors

Dexamethasone is a potent immunosuppressant and NFκB inhibitor used to treat many types of inflammatory diseases and septic shock [129, 130]. Dexamethasone is effective in preventing SEB-induced shock in the "SEB-only" model and the LPS plus SEB model of toxic shock [116,131]. However, inhibition of NFκB is protective in these mouse models only if blockade by dexamethasone is applied early after superantigen exposure and for a long duration. Interestingly, the combined effect of early dexamethasone treatment followed by the anti-oxidant N-acetyl cysteine later is also efficacious in the "SEB-only" murine model of toxic shock [132]. Although the NFκB pathway is an obvious target, other inhibitors of NFκB have only been partially successful in vivo [133] as the NFκB cascade is a major signal transduction pathway for many other cellular receptors including PRRs and cytokine receptors. NFκB is a central

regulator of apoptosis and inflammation and is essential for host defense. Mice with deletion of NFκB genes have abnormal morphogenesis and die shortly after birth.

Calcineurin inhibitors

Cyclosporin A (CsA) and tacrolimus (FK506) are two well-known FDA-approved calcineurin inhibitors used clinically to prevent kidney graft rejection [42]. Both drugs form molecular complexes with their cellular receptors, cyclophilin and FKBP12 (FK506 binding protein 12), respectively, to inhibit the calcium-dependent phosphatase function of calcineurin. Although CsA inhibits SEB-induced T-cell proliferation in vitro, reduces serum cytokines, and attenuates pulmonary inflammation, it has no effect on lethality in non-human primates [134]. In contrast, CsA effectively prevents SEB-induced shock in a D-galactoseamine-sensitized murine model of toxic shock [24]. Tacrolimus suppresses superantigen-induced T-cell activation in vitro but does not reduce lethality in HLA-DR3 transgenic mice [135].

mTORC1 inhibitors

Rapamycin is a well-known mTORC1 inhibitor as it binds to the immunophilin FKBP12, forming a complex which then blocks mTORC1 activation. Rapamycin (also known as sirolimus) is used clinically to prevent kidney transplant rejection as it shows less nephrotoxicity than calcineurin inhibitors [42]. mTORC1 is a central integrator of environmental cues including immune, nutrient and energy signals arising from TCR, costimulatory receptors, growth factors, ATP, glucose and amino acids [47-50]. As described earlier, superantigen induces mTORC1 downstream of PI3K/Akt via the three signals of T-cell activation. More recent studies indicate mTORC1 regulates T-cell differentiation and increases Treg function [136, 137]. Rapamycin inhibits cytokine release and T-cell proliferation by blocking mTORC1 signaling induced by

SEB [124, 138]. Rapamycin protects mice from SEB-induced shock even when administered one day after SEB administration, providing an effective drug post exposure. Inhibition of mTORC1 by rapamycin likely prevents organ damage by inducing autophagy and increasing the numbers of Treg cells, as well as their suppressive functions simultaneously [136, 137].

Although studies using rapamycin to block SEB-induced shock in the "SEB-only" mouse model show that rapamycin is efficacious, the immunological mechanisms have not been fully elucidated. Subsequent study of gene profiling in the same model sheds light on the effects of "pure" SEB without potentiating agents in vivo by revealing damage response, DNA sensors and ISG upregulation upon SEB exposure [92]. Furthermore, the same damage response activators are present in all organs (lung, spleen, liver, kidney, and heart) and mouse PBMC in the presence of SEB without confounding synergistic agents. Rapamycin is also a potent autophagy inducer in addition to its ability to block mTORC1 [139]. Recent studies show mTORC1 regulates T-cell differentiation and its activation blocks Fox3p, a key transcription factor for Treg [137, 140]. Thus rapamycin blockade of mTORC1 likely induces a variety of regulatory pathways in SEB-stimulated cells, including autophagic removal of damaged mitochondria, induction of functional Treg, downregulating apoptosis, inflammatory cytokines and T-cell proliferation. The success of delayed administration of rapamycin in preventing the toxic effects of SEB indicates that the tissue damage from cytokine storm and resolution of inflammation in organs to be critical in preventing shock.

Conclusion

The host response to superantigen initiated by cellular activation of monocyte /macrophages and T-cells leads to the early release of IFNs, inflammatory cytokines and chemokines. IFNs induce many genes regulating NFκB and apoptosis. Inflammatory cytokines such as TNFα and IL-1

cause tissue damage by activating pathways leading to NFκB, MAPK cascades and apoptosis. The excessive T-cell proliferation and enhanced protein synthesis driven by superantigens also induce ER stress, ROS and MAPK cascade. Both extrinsic and intrinsic pathways of apoptosis are induced in vitro and in mouse models of superantigen-induced toxic shock. This uncontrolled superantigen-induced apoptosis is promoted by the upregulation of multiple caspases, CARD, TNFR1, CD95 and other pro-apoptotic molecules. The damage response induced by superantigens starts with effects from inflammatory cytokines and apoptotic programs activated by IFNs and TNFα. DAMPs such as mitochondrial ROS and mtDNA trigger additional apoptosis, activate inflammasomes, and induce IRFs and other transcription factors for ISGs. Inflammation, apoptosis, and cellular damage from superantigen activation lead to tissue injury and organ dysfunction. The simultaneous induction of PI3K/mTORC1 in superantigen-activated cells blocks autophagy, resulting in inflammasome activation, accumulation of damaged mitochondria and uncontrollable damage in multiple organs. FDA-approved immunosuppressants directed at inhibiting mediator release and the downstream cell destructive events provide proof of concept that these drugs can be transitioned to clinical use against superantigens.

Disclaimer

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Table 1. FDA-approved immunosuppressants tested for efficacy in animal models of superantigen-induced toxic shock

Pharmacologic agent	**Mechanism**	**Biological effects against SEB**
CTLA4-Ig	Blocks costimulatory receptor CD28	Blocked binding of CD28 to CD80/86 and attenuated TSST-1-induced TNFα, and IFNγ [123]. Protected 75% of mice from TSST-1-induced toxic shock [123].
Dexamethasone	Inhibits NFκB	Inhibited TSST-1-induced proinflammatory cytokines and chemokines in human PBMC [52]. Reduced serum levels of cytokines, attenuated hypothermia due to SEB, and protected mice 100% in both SEB-induced and SEB plus LPS-induced shock models [116, 131].
Cyclosporin A	Binds cyclophilin, inhibits calcineurin phosphatase and T-cell activation	Blocked SEB-induced cytokines and proliferation. Protected mice from shock in SEB plus galactoseamine model [24]. Blocked cytokines and T-cell proliferation but had no effect on lethality in non-human primates [134].
Tacrolimus (FK506)	Binds FKBP12, inhibits calcineurin phosphatase and T-cell activation	Suppressed serum cytokines but provided no protection against SEB-induced shock in HLA-DR3 transgenic mice [135].
Rapamycin (sirolimus)	Binds FKBP12, inhibits mTORC1 and induces autophagy.	Blocked SEB-induced cytokines, chemokines and T-cell proliferation. Protected mice 100% from lethality even when administered 24 h after SEB [124].

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